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14. ABSTRACT
The three goals of this project are: (i) to discover and develop novel drugs which could prevent or reverse the vascular leak syndrome; (ii) to develop angiogenesis inhibitors which would inhibit post-operative abdominal adhesions; and, (iii) to isolate endothelial progenitor cells from blood, capable of being expanded in vitro and applied to vascular grafts. Progress has been made in each category: we are elucidating the mechanism of Caplostatin, one of the most potent anti-vascular leak drugs; we have been able to prevent intra-abdominal adhesions effectively by the use of the anti-angiogenic activity of celecoxib; and, we have identified conditions for expanding endothelial progenitor cells and smooth muscle progenitor cells obtained from umbilical cord blood so that they can eventually be grown in vascular grafts.

15. SUBJECT TERMS
endostatin; Caplostatin; angiogenesis; angiogenesis inhibitors; celecoxib; adhesions; circulating endothelial cells; endothelial progenitor cells; mesenchymal stem cells; tissue-engineering

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Project I: New Therapy for Vascular Leak Syndromes

Judah Folkman, M.D., ***Principal Investigator***
Carmen Barnes, Ph.D., ***Research Associate***
Kashi Javaherian, Ph.D., ***Research Associate***
Sandra Ryeom, Ph.D., ***Research Associate***
Tong-Young Lee, Ph.D., ***Research Fellow***
Daniela Prox, M.D., ***Research Fellow***

I: INTRODUCTION:

We have made progress in trying to elucidate the mechanism of Caplostatin. This conjugate fumagillin analog TNP-470 with the copolymer N-(2-hydroxypropyl)methacrylamide (HPMA) is an angiogenesis inhibitor that is among the most potent anti-vascular leak molecules.

II: BODY:

An ongoing experiment is to determine if anti-vascular leak activity of Caplostatin depends on its interaction with endostatin and/or thrombospondin-1. Endostatin and thrombosponsi-1 are endogenous angiogenesis inhibitors normally found in the circulation at low level. We will induce vascular leak by subcutaneous injection of cytokines such as, VEGF, PAF (platelet activating factor), bradykinin, serotonin, histamine, and protein M, in mice genetically deficient for thromobospondin-1 or endostatin. Before injecting these permeability-inducing cytokines, we will inject Evans blue dye intravenously (1). This binds to albumen, which leaks into a subcutaneous site, previously injected by one of the cytokines mentioned above. The site of injection turns blue because of leakage of blue-stained albumen (the Miles test). We have previously reported that Caplostatin reverses the Miles test so that no blue dye appears at the site of injection of vascular leak molecule. If Caplostatin is dependent upon thrombospondin-1 or endostatin for its vascular leak activity, we would expect that the Miles test would not be abrogated by administration of Caplostatin.

We have not yet carried out gene array profiles of conditioned media of endothelial cells treated with Caplostatin. However, we will try to do this in the next quarter.

To develop a more rapid bioassay to test inhibitors of the vascular leak syndrome we will employ the chick embryo chorioallantoic membrane to display the Miles test. In this system three-day-old chick embryos are removed from their shell and each is cultivated in a single petri dish. The vascular chorioallantoic membrane grows from a diameter of 1 cm on day 5 to at least ~5 cm diameter by 10-12 days. We are able to evaluate up to 50 chick embryos a day in which the Evans blue has been injected intravenously and then the permeability inducing molecule is placed on top of the chorioallantoic membrane in a Matrigel pellet, or is injected into the membrane with an ultra-thin #30 hypodermic needle.

Endostatin. We have demonstrated that therapy of tumor-bearing mice with endostatin protein or with endostatin gene therapy, both reveal a biphasic (U-shaped) dose-efficacy curve of antiangiogenic and anti-cancer activity. We have published this data in two recent reports (2,3).

Previously, we demonstrated that the entire anti-angiogenic activity of endostatin can be mimicked by a 25-amino acid peptide located at the N-terminus of endostatin. There is an atom of zinc bound to endostatin which is incorporated by the peptide. Removal of zinc gives rise to a peptide without anti-tumor activity.

We have investigated the endostatin peptides in two other diseases. Endometriosis, the ectopic growth of endometrial tissue, is an estrogen driven disease, affecting women during child-bearing age. The endostatin peptides discussed before were tested in a mouse model of endometriosis. The N-terminus peptide and an internal peptide suppressed growth of tumor significantly in vivo. However, estrogen cycling and corpus luteum formation were normal in both groups. No titration of peptides was carried out to determine the relative potency of the two peptides (4).

Retention of lipoprotein by proteoglycans in the subendothelial matrix is an early event in atherosclerosis. We found out that endostatin is differentially depleted in blood vessels affected by atherosclerosis. By testing different endostatin peptides to determine which peptide is responsible for this phenomenon, we found a peptide next to the N-terminus peptide that contains an alpha-helix structure and is responsible for the ability of endostatin peptide to prevent retention of lipoproteins and their binding to proteoglycans in the sub-endothelial matrix (5).

We described in the last progress report that a polymer called HPMA (N-(2-hydroxypropyl)methacrylamide) has been cross-linked to the peptide. Mice with Lewis-lung carcinoma (LLC) were treated with this conjugate. The preliminary data show a substantial increase in anti-tumor activity of the conjugate compared with the peptide alone. We are now planning to test the endostatin peptide in a wide spectrum of mouse tumors, and also in animal models with vascular leak syndrome. The latter will include pulmonary edema induced by interleukin-1, in collaboration with Judah Folkman in this project.

As mentioned in our quarterly reports, Ronit Satchi-Fainaro, Ph.D., was offered an Associate Professorship at Tel Aviv University in Israel, and in October 2005 left the laboratory to take this prestigious position. We have made plans for her to spend two to three months each summer in our laboratory *(this will not be charged to the DOD).* In the meantime, Carmen Barnes, Ph.D., has taken over the Caplostatin project. Carmen is an outstanding biochemist. We are also interviewing postdoctoral fellows with a background in polymer chemistry to increase the strength of this project. Sandra Ryeom, Ph.D., who is an Instructor at Harvard Medical School and in the Lab full-time, has joined this project to work on the mechanism of the vascular leak protection by Caplostatin and endostatin.

Ongoing Work:

A. In the next year we will extend our studies with Caplostatin as an inhibitor of the vascular leak syndrome in larger animals (rats and rabbits), which will include obtaining the appropriate animal protocols and approvals.

B. A fluorescent label (fluorescein isothiocyanate) has been conjugated to the N-terminus peptide and also to the full-length endostatin. We will inject mice with these reagents and follow their binding sites *in vivo*. HPMA-hP1 conjugate will be tested for anti-tumor activity more extensively.

C. Future plans include the assessment of vascular permeability/leakage after treatment of the TSP-1 null mice and wildtype mice with endostatin. We will utilize the Miles assay to determine vascular permeability with various agonists including IL-1. Furthermore, our plans include treatment of both TSP-1-null and collagen XVIII-null mice with TNP-470 and its polymer conjugate, Caplostatin. These experiments will determine whether the mechanism of inhibition of vascular leak may be due to the upregulation of these endogenous angiogenic regulators. We will compare the ability of these compounds to suppress vascular permeability in the wildtype and TSP-1 null and collagen XVIII-null mice.

III: KEY RESEARCH ACCOMPLISHMENTS:

- Developing methods to scale up production of Caplostatin, so that we can eventually work with a biotech company willing to manufacture it with the goal of clinical trials.

- Caplostatin synergized with Avastin (an FDA-approved angiogenesis inhibitor that blocks circulating VEGF), to eradicate 50% of human colorectal carcinomas in SCID mice and to permanently inhibit the other 50% at a microscopic dormant size.

- A 25-amino acid peptide located at the N-terminus of endostatin retained all of the antiangiogenic and anti-tumor activity of the full-length protein. We showed that the peptide also effectively inhibited endometriosis in the mouse model (5). Endostatin peptide prevents retention of lipoproteins and their binding to proteoglycans in the sub-endothelial matrix (which is an early event in atherosclerosis).

- The endostatin peptide was cross-linked to the HPMA polymer and revealed a significant increase in anti-tumor activity of the conjugate compared to the peptide alone.

IV: REPORTABLE OUTCOMES:

Invited Review by Experimental Cell Research:
Folkman, J., Experimental Cell Research. Antiangiogenesis in cancer therapy-endostatin and its mechanisms of action. 2006 in press (publication March 12, 2006).

Presentation:
"Broad Spectrum Angiogenesis Inhibitors," Dr. Judah Folkman, American Association for Cancer Research meeting, Boston, MA, November 12, 2005

V: CONCLUSIONS:

a. We are elucidating the mechanism of Caplostatin and developing new methods to scale up its production so that all of this information can be utilized to make Caplostatin available for clinical trial. Once the drug is approved by the FDA for cancer, it may be very useful for the military as an anti-vascular leak drug.

We are studying the anti-vascular leak activity of endostatin and its short peptide. Because endostatin is normally in the circulation, one goal of this project will be to determine if endostatin can be elevated in the blood sufficiently to prevent or reverse the vascular leak syndrome. For example, it has been previously reported by others (6) the administration of low-dose celecoxib will elevate circulating endostatin approximately two-fold. Ragu Kalluri at the Beth Israel Deaconess Hospital, Harvard Medical School, has recently discovered that orally administered doxycycline will also elevate endostatin in the blood (Kalluri 2006, unpublished).

b. "So What:" If novel non-toxic drugs can be developed that can be administered to a soldier after blast injuries or asphyxiation, which induce pulmonary edema, it may be possible to prevent or to reduce this dangerous syndrome. Furthermore, if a soldier entering combat could take a single dose of a relatively non-toxic drug that would increase circulating endostatin, this novel approach might prevent pulmonary edema from a blast injury or asphyxiation.

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Project II: The Prevention of Post-operative and Traumatic Abdominal and Non-abdominal Adhesions

Mark Puder, M.D., Ph.D., ***Principal Investigator***
Sendia Kim, M.D., ***Research Fellow***

I. INTRODUCTION:

Several approaches have been attempted to inhibit intra-abdominal adhesion formation, most with limited success [1,2]. While most studies studying the prevention of post-operative adhesion formation have focused on promoting fibrinolysis and inhibiting re-epithelialization of peritoneal surfaces, several investigators have implicated mediators of angiogenesis in the formation of adhesions. Because newly forming adhesions produce angiogenic factors that recruit new endothelium, COX-2 inhibitors are likely to target the expressed COX-2 enzyme on the newly proliferating vasculature. Vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor beta are up-regulated during adhesion formation [3-5]. Because newly forming adhesions produce angiogenic factors that recruit new endothelium, COX-2 inhibitors are likely to target the expressed COX-2 enzyme on the newly proliferating vasculature. This effect was demonstrated by the significant decrease in microvessel density in the celecoxib treated animals from our murine model study [6]. In addition results of our previous study in a murine model demonstrated that celecoxib led to a more complete reduction of adhesions than another COX-2 inhibitor, rofecoxib. This was thought to be due to the additional antifibroblastic and anti-inflammatory activity of celecoxib [7].

A rabbit model may be more predictive of human disease and are the smallest large animals that have been used in testing adhesions. We will use an adhesion model in rabbits to test the effects of celecoxib in adhesion prevention.

II. BODY:

Adhesion model 1: Twelve female New Zealand white rabbits weighing 2.5 kg were pre-medicated with glycopyrrolate and buprenorphine, induced with 3-4% isoflurane and placed under general endotracheal anesthesia. A 5 cm midline incision was made in the lower abdomen. The cecum/sacculum rotundum was abraded with an OR towel until punctate hemorrhages appeared. The bowel was then placed back into the abdominal cavity. A 3x3 cm incision was made on the peritoneum of the right lateral abdominal wall. The peritoneal lining was dissected off the abdominal wall. Pressure was held until all bleeding stopped. The abdominal wall was then closed.

Drug treatment: The rabbits were divided into 2 groups. The control group received applesauce alone. The treatment group received celecoxib mixed in applesauce at a dose

of 75mg/kg/day, divided twice daily, beginning on the day before surgery and for 10 days post operatively.

Quantification of Intra-abdominal adhesions: Ten days after surgery, the rabbits were euthanized and adhesion formation was measured by two blinded individuals based on a previously described scoring system [8]. The tenacity, type, and extent of adhesions to the deperitonealized surface, as well as the extent of adhesions to the cecum were graded on a scale of 0 to 4 (table 1). The total adhesion score was the sum of the four individual scores. A minimum score was 0 and the maximum score was 16. Tenacity was rated as none (0), adhesions fell apart (1), lysed with traction (2), lysed with blunt dissection (3), lysed with sharp dissection (4). Type was scored as none (0), filmy (1), mildly dense (2), moderately dense (3), very dense (4). Extent was measured as the percent of the deperitonealized surface of the abdominal wall covered by adhesion: 0% (0), <25% (1), 25-50% (2), 50-75% (3), >75% (4). Cecal adhesions were graded as none (0), adhesions fell apart (1), lysed with traction (2), lysed with blunt dissection (3), lysed with sharp dissection (4).

Preliminary data from model 1: Only one control rabbit developed adhesions and no celecoxib animals developed adhesions. Its adhesion score was 11 (tenacity 4, extent 4, cecal adhesion tenacity 3).

Adhesion model 2: Due to the lack of adhesion formation in the control group we made serosal cuts into the bowel wall, a technique which has been previously described [9]. The abdomen was entered and a 3x3 cm incision was made on the right lateral abdominal wall with removal of the peritoneum in this area, exposing the underlying muscle. Ten serosal cuts were placed on the cecum. The remaining cecum was abraded with an OR towel until punctate hemorrhages appeared and the abdominal wall was closed.

Quantification of Intra-abdominal adhesions: as above

Preliminary data from model 2:

No.	Celebrex	Tenacity	Extent	Cecal	Total
431	Yes	4	4	2	10
433	Yes	4	4	0	8
443	Yes	3	4	0	7
444	Yes	3	4	0	7
445	Yes	4	4	0	8
435	No	4	4	2	10
437	No	4	4	4	12
439	No	4	4	4	12
446	No	4	4	0	12

There was extensive adhesion formation in all the rabbits. This model appeared to be more of a wound healing experiment rather than an adhesion model. We therefore eliminated the sidewall excision.

Adhesion model 3: The entire cecum was then abraded with an OR towel until punctate hemorrhages appeared. Ten serosal cuts were made on the cecum. Small enterotomies were made in rabbits 448, 449, 450, 452, 459, 489, 498 and closed with sutures. Rabbit 451 had a bowel rupture in which 75% of the bowel circumference was opened and subsequently repaired. The remaining rabbits did not have enterotomies.

Quantification of Intra-abdominal adhesions: Scoring of adhesions to the abdominal wall "patch" (the deperitonealized area) was replaced by tenacity of adhesions to the midline based on the same scale. The extent of adhesions to the patch was entirely eliminated.

Preliminary data from model 3: Two rabbits (452, 498) expired during the study. Necropsy revealed torsion of the bowel resulting in ischemia and perforation in one rabbit and contained perforation in the other.

See table, next page.

No.	Celebrex	Midline adhesion	Cecal adhesion
448	No	0	Cecal-cecal 3
449	No	4	Cecal-cecal=3 Cecal-appendix=3 Cecal-SI=4 Cecal-LI=4
459	No	0	Cecal-appendix=2 cecal-cecal=4 Cecal-LI=4 Cecal-omentum=1
456	No	0	Cecal-omentum=2 Cecal-SI=4 Cecal-LI=3 Cecal-cecal=3
457	No	0	Cecum-colon=2
458	No	0	Cecum-cecum=4 Cecum-LI=4
486	No	0	Cecal-cecal=4
488	No	0	Cecal-cecal=4 Cecal-sacculum=4 Cecal-LI=4
490	No	0	Cecal-cecal=3 Cecal-sacculum=3 Cecal LI=4
495	No	3	(cecal-midline=3)
497	No	0	Cecal-cecal=4
499	No	0	Cecal-LI=4 Cecal-cecal=4
450	Yes	0	Cecum-cecum=4
451	Yes	0	0
453	Yes	0	Cecum-cecum=4 Cecum-SI=4
454	Yes	0	Cecum-cecum=4 Cecum-SI=3
455	Yes	0	Cecum-SI=2 Cecum-LI=4
487	Yes	0	0
489	Yes	0	Cecal-cecal=3
491	Yes	0	0
494	Yes	0	Cecal-stomach=3 Cecal-LI=2 Cecal-cecal=4
496	Yes	0	0

Although there appears to be a trend of decreased adhesion formation in the rabbits that have been treated with celecoxib, the serosal injury model is not an ideal model for several reasons. Due to the thinning of the bowel wall, three of the rabbits (453, 454, 494) developed intraabdominal perforations post operatively, possibly confounding the effect of the celecoxib. In addition, we have found this model to be extremely challenging to replicate as the procedure requires thinning the bowel wall resulting in intraoperative perforation. The perforation, fecal contamination, and repair suture may all have an effect on adhesion formation.

Adhesion model 4: We are now working on a uterine horn model. Retired female rabbits will be divided into treatment and control groups. A low midline incision will be made and the uterus will be delivered. The uterus and the uterine horns will be abraded with a 10 blade scalpel until punctate hemorrhages appear. Collateral blood supply within the mesosalpinx and mesoovarium will be tied off on each side of the uterus. The uterus will be placed back into the abdomen and the abdomen closed.

Quantification of Intra-abdominal adhesions: Scoring of adhesions to the sidewall defect was replaced by tenacity of adhesions to the midline incision based on the same scale.

Preliminary data from model 4: A small pilot study of 3 rabbits revealed successful adhesion formation in all three rabbits. None of these rabbits were treated with celecoxib. We are planning to proceed with testing this model.

III. KEY RESEARCH ACCOMPLISHMENTS:

- Revision of adhesion scoring.
- Development of intra abdominal adhesion model in rabbits.

IV. REPORTABLE OUTCOMES: None

V. CONCLUSIONS:

Several techniques have been attempted for developing a rabbit model of adhesions. Our initial experiment did not result in the formation of adhesions. Although we modified our technique several times, we were unsuccessful in developing a reliable adhesion model. Although results in our third model demonstrated a trend towards decreased adhesion formation, this was inconsistent due to the confounding factors of intra-operative and post-operative perforations. We are now working on a uterine horn model, which appears to result in adhesion formation and is easy to replicate.

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Project III: Creating New Blood Vessels using Blood-derived Endothelial and Mesenchymal Progenitor Cells

Joyce Bischoff, Ph.D., ***Principal Investigator***
Juan Martin-Melero, Ph.D., ***Research Fellow***

I: INTRODUCTION:

The hypothesis of this project is that autologous human endothelial progenitor cells and mesenchymal cells from adult peripheral blood can be isolated, expanded, and used to create blood vessels and microvascular networks as needed for tissue engineered organs and tissues, and possibly for repair of damaged tissue. Using sheep as a model system, we showed that endothelial progenitor cells isolated from a small amount of peripheral blood can be used to create non-thrombogenic long-lasting functional blood vessels (Kaushal et al., 2001). This provided proof-of-principal for our hypothesis and encouraged us to develop methods for isolating endothelial progenitor cells from human blood, and additionally mesenchymal stem cells, that we postulate can be used to form the smooth muscle layer in tissue engineered blood vessels. Due to the inherent heterogeneity of blood preparations, both phenotypical and functional characterization, as well as the evaluation of cell expansion capability, for both endothelial and mesenchymal progenitors must be performed. Our goal in this project is to define the most straightforward and robust methods for isolating and expanding sufficient quantities of these cells from adult peripheral blood and to test their ability to form blood vessels that can form anastamoses (i.e., connections) with blood vessels in a living mouse.

II: BODY:

Technical Objective 1: To apply the methods we developed for isolating endothelial progenitor cells and mesenchymal stem cells from human cord blood to adult peripheral blood to determine feasibility of isolating autologous cells from a soldier's own blood

We showed previously that human endothelial progenitor cells can be isolated from cord blood by selecting for cells co-expressing a stem cell marker, CD133, and an endothelial marker, CD34 (Wu et al., 2004). This procedure yields highly purified, undifferentiated cells that require 3-4 weeks of in vitro culture before the cells begin to grow. After 28 days, approximately 200,000 endothelial progenitor cells-derived endothelial cells can be obtained. To reduce cell loss and to capture all endothelial cells with high proliferative potential, we began modifying the two-step selection procedure and instead, simply plated the mononuclear cell fraction from human umbilical cord blood, allowed the cells to grow in a culture medium designed for endothelial cells, and then purified the endothelial cells by selecting for CD31+ cells. In this procedure, we were able to obtain 10^{11} endothelial cells after 28 days – an increase of five orders of magnitude over what was achieved in our previous work (Wu et al., 2004). The endothelial phenotype of these cells was confirmed by

FACS, indirect immunofluorescence and RT-PCR. Endothelial progenitor cells were uniformly positive for CD31, VE-cadherin, vWF, CD105, CD146, KDR and eNOS, while negative for the hematopoietic and monocytic markers CD45 and CD14. The potential of these endothelial progenitor cells for vasculogenic or angiogenic function was demonstrated by the superior proliferative and migratory responses to VEGF and bFGF when compared to human dermal microvascular endothelial cells. The endothelial progenitor cells were routinely expanded for more than 65 population doublings. As the endothelial progenitor cells were expanded in culture, the morphology, growth kinetics and proliferative responses toward angiogenic growth factors progressively resembled those of mature human microvascular endothelial cells, indicating a process of in vitro cell maturation over time. We hypothesize that endothelial progenitor cells at different stages of ex vivo expansion will be required for specific tissue-engineering applications depending on the functional and phenotypic properties needed to achieve the appropriate degree of vascular development.

In summary, using cord blood as a model system, we have gained valuable insights on the growth and expansion capabilities of human endothelial progenitor cells. Our goal to transfer these methodologies to endothelial progenitor cells from adult peripheral blood has proved more difficult than we anticipated. We have obtained endothelial cells from adult peripheral blood but could only expand the cells 20-25 population doublings, which is in contrast to the 65-70 population doublings achieved with cord blood-derived endothelial progenitor cells. We have applied several modifications to our procedure, such as depleting monocytes from the mononuclear cell fraction, without success. We are currently testing if the addition of human plasma or increased concentrations of exogenous vascular endothelial growth factor will yield endothelial cells with high proliferative potential. We are still confident, based on the literature (Ingram et al., 2005), that we can achieve the goals in this technical objective.

For mesenchymal/smooth muscle progenitor cells from blood, we have developed a simple procedure in which cells expressing three different smooth muscle markers can be isolated from cord blood by simply changing from an endothelial growth medium to one suitable for growth of mesenchymal progenitor cells: DMEM, 10% fetal bovine serum, 1% non-essential amino acids and glutamine/penicillin/streptomycin. These cells are currently being characterized for growth and differentiation potential.

Technical Object 2: <u>To determine if mesenchymal stem cells co-seeded with endothelial progenitor cels will induce microvessel formation in biodegradable scaffolds.</u>

Preliminary experiments are underway using a polyhydroxyalkanoate (PHA) non-woven scaffold that we have shown is suitable for adhesion and growth of endothelial cells (unpublished data).

Technical Object 3: To implant tissue-engineered microvascular networks into immune-compromised mice and determine if the tissue-engineered microvessels will form functional anastomoses with host vessels.

These experiments will be pursued in the coming year.

III: KEY RESEARCH ACCOMPLISHMENTS:

- Identified a simple and economical procedure for dramatically increasing the yield of highly proliferative endothelial cells from cord blood.

- Identified measurable features of endothelial progenitor cell maturation *in vitro*: increase in doubling time, decrease in response to angiogenic factors, increase in cell size and reduced intensity of cell surface marker CD34.

- Identified simple cell culture conditions for promoting growth of mesenchymal/smooth muscle progenitors from cord blood.

IV: REPORTABLE OUTCOMES:

Abstracts:
Characterization of blood derived endothelial progenitor cells for tissue engineering applications, Juan Melero-Martin, Zia Khan, Sailaja Paruchuri, Xiao Wu and Joyce Bischoff. To be presented at the 10th Biennial Meeting of the International Society for Applied Cardiovascular Biology, March 8-11, 2006, La Jolla, CA

Presentations:
Blood-derived Endothelial Progenitors, Joyce Bischoff, Boston Angiogenesis Meeting 2005, November 8, 2005

Endothelial Progenitors for Cardiovascular Tissue Engineering, Joyce Bischoff, University of Chicago, Committee on Cell Physiology Seminar Invitation, Chicago, IL January 13, 2006

Endothelial Progenitors for Creating Blood Vessels, Joyce Bischoff, CIMIT Forum, Massachusetts General Hospital, Boston, MA, February 14, 2006

V: CONCLUSIONS:

a. During this first year of the project, we have developed a simple procedure for expanding endothelial cells from human umbilical cord blood; the cells grow rapidly, dividing every 17-20 hours, with robust response to angiogenic factors, such that the yield of endothelial cells should be more than a sufficient for creating tissue-engineered arteries. We have shown that the cells express high levels of CD34, a cell surface proteoglycan; and that this can be used to distinguish the cells from mature endothelial cells. We have also identified parameters such as cell size and response to angiogenic growth factors that also serve as markers of endothelial maturation. What has been difficult is to transfer our results to adult

peripheral blood. This difficulty is not unexpected because there are 15-20-fold fewer endothelial progenitor cells in adult blood compared to cord blood. There is also discussion in the literature that the growth potential of cord blood endothelial progenitor cells may differ from adult endothelial progenitor cells (Ingram et al., 2005). This does not undermine our goal to use endothelial progenitor cells from adult blood for cardiovascular tissue-engineering because 1) the proliferative potential could be 2-3 orders of magnitude lower and we would still be able to obtain sufficient numbers of cells and 2) there is evidence that additional VEGF is needed for adult endothelial progenitor cells so simply supplementing with higher concentrations might be sufficient. We are confident we will be successful in isolating endothelial progenitor cells from adult blood in the next year.

The implications of the results we have obtained using cord blood to study the in vitro maturation of endothelial progenitor cells are manifold. First, we know how the cells change as they are expanded in culture: the cell surface expression of CD34 goes from "bright" to "dim" as measured by flow cytometry, the proliferative response to angiogenic growth factors is reduced, and the cytoplasmic volume increases. We speculate that cells at different stages of maturation will be best suited for specific cardiovascular tissue-engineering applications. For example, cells that respond robustly to VEGF and bFGF might be best suited for applications in which de novo vascularization of a tissue is required whereas cells with a more tempered response to angiogenic factors might be best suited for replacing endothelium in injured vessels or for vascular grafts.

b. The second implication of this work is that we may be able to use the CD34 "bright" property of the youngest endothelial progenitor cells to determine number of endothelial cells with high proliferative potential in a small blood sample. Blood samples would be tested for CD34"bright"/CD146 positive cells. (CD146 is an endothelial marker). This is superior to using CD133/CD146 because CD133 is a stem cell marker that is expressed transiently on endothelial progenitor cells. Thus, relying on CD133 for selecting or quantitating endothelial progenitor cells in blood will underestimate the number of endothelial cells with high proliferative potential. The level of CD34 "bright"/CD146 positive cells could be used to assess the suitability of a patient's blood for isolation of autologous endothelial progenitor cells for blood vessel construction or repair. Perhaps CD34"bright"/CD146 positive cells could be isolated directly from the blood by apheresis, allowing collection of these cells from larger volumes of blood if needed.

c. The third implication for this work is that CD34"bright" versus CD34"dim" endothelial cells from peripheral blood – either cord blood or adult blood – can be subjected to gene or protein expression profiling to advance our knowledge of these cells. A practical outcome of this would be the identification of increasingly specific markers that could be used to isolate and/or quantitate cells and to identify new mechanisms to control growth and differentiation for therapeutic applications that will improve the health and well-being of soldiers and their families.

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VII: APPENDICES:

Project I:

1. Folkman, J., Experimental Cell Research. Antiangiogenesis in cancer therapy-endostatin and its mechanisms of action. 2006 in press (publication March 12, 2006).

Project II:

None

Project III:

1. Characterization of blood derived endothelial progenitor cells for tissue engineering applications, Juan Melero-Martin, Zia Khan, Sailaja Paruchuri, Xiao Wu and Joyce Bischoff. To be presented at the 10th Biennial Meeting of the International Society for Applied Cardiovascular Biology, March 8-11, 2006, La Jolla, CA.

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Review Article

Antiangiogenesis in cancer therapy—endostatin and its mechanisms of action

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Abstract

The first angiogenesis inhibitors for cancer have now been approved by the F.D.A. in the U.S. and in 28 other countries, including China. The majority of these are monotherapies that block VEGF. However, mutant tumor cells may over time and produce redundant angiogenic factors. Therefore, for long-term use in cancer, combinations of angiogenesis inhibitors or broad spectrum angiogenesis inhibitors will be needed. The two most broad spectrum and least toxic angiogenesis inhibitors are Caplostatin and endostatin. Endostatin inhibits 65 different tumor types and modifies 12% of the human genome to downregulate pathological angiogenesis without side-effects. The recent discovery that small increases in circulating endostatin can suppress tumor growth and that orally available small molecules can increase endostatin in the plasma suggests the possible development of a new pharmaceutical field.

Keywords: Endostatin; Angiogenesis inhibitors

Contents

Introduction

Angiogenesis inhibitors for the treatment of cancer have now been approved by the F.D.A. in the U.S. and in 28 other countries, including the European Union (Table 1). In December 2003, when thalidomide was approved in Australia for the treatment of multiple myeloma, Gareth Morgan, Chair of the U.K. Myeloma Forum Scientific Subcommittee, said "It is the best treatment advance in 25 years and people are doing well with it" [1]. When Avastin

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Table 1
Angiogenesis inhibitors approved by the FDA in the U.S. and in 28 other countries (including endostatin (China) and Macugen (U.S. and Brazil)

Date approved	Drug	Place	Disease
December 2003	Thalidomide	Australia	Multiple myeloma
February 2004	Avastin	U.S. (FDA)	Colorectal cancer
November 2004	Tarceva	U.S. (FDA)	Lung cancer
December 2004	Avastin	Switzerland	Colorectal cancer
December 2004	Macugen	U.S. (FDA)	Macular degeneration
January 2005	Avastin	European Union (25 countries)	Colorectal cancer
September 2005	Endostatin (Endostar)	China (SFDA)	Lung cancer

was approved in the U.S., for the treatment of colorectal cancer in February 2004, Mark McClellan, the Director of the FDA, stated that, "Angiogenesis inhibitors can now be considered as the fourth modality of cancer therapy." In May 2004, Andrew C. von Eschenbach, Director of the National Cancer Institute, and Allen M. Spiegel, Director of the National Institute of Diabetes and Digestive and Kidney Disease, said, "We can unequivocally say that angiogenesis is not only a critical factor for cancer, but for a host of other diseases" [2]. In September 2005, endostatin (Endostar) was approved by the State FDA in China for the treatment of non-small-cell lung cancer [3]. In addition, other drugs previously approved by the FDA for other uses have recently been found to have antiangiogenic activity. These include doxycycline [4,5], zolendronate [6], rosiglitazone [7], celecoxcib [8], and low-dose interferon alpha [9,10] Furthermore, certain other anti-cancer drugs that were originally designed to inhibit a growth factor receptor, (i.e., Tarceva [11]), or to inhibit proteosomes (i.e., Velcade [12]), have also shown various degrees of antiangiogenic activity. At least 30–40 angiogenesis inhibitors are currently in pre-clinical or clinical trials. In addition to cancer therapy, the FDA recently approved Macugen [13] (pegaptanib), an aptamer of VEGF, for the treatment of macular degeneration.

Therefore, after more than three decades of research on angiogenesis, the translation of antiangiogenic therapy from laboratory to the clinic is underway and is robust. It is now possible to think about new opportunities which this novel class of drugs may permit for the treatment of cancer and other angiogenesis-dependent diseases. We can also ask about new directions in research that may further improve antiangiogenic therapy in the future.

Antiangiogenic monotherapy

The first FDA-approved angiogenesis inhibitor, Avastin (bevacizumab), blocks a single angiogenic protein, VEGF, produced by about 60% of human tumors. It was the first angiogenesis inhibitor to demonstrate significant prolongation of survival in colon cancer [14]. Significant increase in survival has also been demonstrated for lung cancer and breast cancer by antiangiogenic therapy (personal communication, Dr. Roy Herbst, in a Presidential Symposium on Angiogenesis at the American Society of Oncology, May 18, 2005).

However, advanced stages of breast cancer can express up to six proangiogenic proteins [15]. In advanced stages of neuroblastoma, high expression of seven angiogenic proteins is found [16]. Human prostate cancer can express at least four angiogenic proteins, including VEGF, bFGF, IL-8 and PDGF [17]. If these findings extend to other human cancers, as survival increases, it is likely that residual tumors in a given patient could express redundant angiogenic factors. Such tumors could become refractory to an angiogenesis inhibitor that blocks a single angiogenic factor. The result would simulate "acquired drug resistance" of tumor cells to cytotoxic chemotherapy. Other hypothetical mechanisms for resistance to antiangiogenic therapy are discussed in [18]. However, the possibility that a tumor could, over time, produce redundant angiogenic factors not matched by the angiogenesis inhibitor may already be problematic. Therefore, "resistance" to antiangiogenic therapy differs from tumor resistance to cytotoxic chemotherapy and may be preventable.

To prevent such a potential refractory state, combinations of angiogenesis inhibitors are already being used to broaden their therapeutic efficacy. These approaches include Avastin plus Tarceva or Avastin plus antiangiogenic chemotherapy [19] (metronomic chemotherapy) [20]. Furthermore, another class of angiogenesis inhibitors (i.e., Sugen11248 [21]) that can counteract three angiogenic proteins, VEGF, bFGF and TGF-alpha, is currently in clinical trial.

In the coming years, however, it is likely that *very broad spectrum* antiangiogenic therapy will be desirable, especially to facilitate a goal of "converting cancer to a chronic manageable disease" [22].

Broad spectrum antiangiogenic therapy

But, there are obstacles to the development of drugs with a broad spectrum of antiangiogenic activity. The conventional wisdom about a cytotoxic chemotherapeutic drug is that the more narrowly focused its target (i.e., "smart drug"), the more likely that the drug will be more active against a tumor and less toxic to normal tissues. Gleevec illustrates this concept, but it also reveals that a narrow target may lead to rapid onset of drug resistance. In contrast, there is a widely held belief that 'broad-spectrum' anti-

cancer drugs with 'multiple targets' will generate many side-effects. This may be true for cytotoxic anti-cancer drugs, but not for certain angiogenesis inhibitors.

For example, of the *synthetic* angiogenesis inhibitors, TNP-470 [23], a synthetic analogue of fumagillin, has an anti-cancer spectrum in pre-clinical studies that is broader than virtually any other anti-cancer drug. When TNP-470 is conjugated to a co-polymer (HPMA) to form Caplostatin [24], it has little if any toxicity over a broad range of effective doses.

Of the *endogenous* angiogenesis inhibitors in the body, endostatin has the broadest anti-cancer spectrum. It targets angiogenesis regulatory genes on more than 12% of the human genome [25], and yet it is the least toxic anti-cancer drug in mice. In humans, endostatin has virtually no toxicity and has revealed no resistance even when it has been administered to patients every day for up to >3.5 years without interruption. Endostatin, therefore, represents a model of a broad spectrum angiogenesis inhibitor that in the future could be a platform anti-cancer agent for co-administration with other therapies. The most important contribution of endostatin may be that it could introduce a shift in conventional thinking from the development of angiogenesis inhibitors for narrowly focused targets, toward angiogenesis inhibitors with multiple angiogenesis regulatory targets, but little or no toxicity. These two classes of angiogenesis inhibitors, 'focused target' and 'broad spectrum target', are not mutually exclusive and may be administered together. Because endostatin has recently been approved in China for non-small-cell lung cancer (Endostar), we may soon learn from the Chinese if this concept is validated.

Finally, one of many unexpected outcomes of the study of endostatin by hundreds of investigators (in 770 reports at this writing), over the 8 years since the first report of its discovery in 1997, is that it can be elevated in the blood by small, orally available molecules (see below). This reveals the possibility of the emergence of an entirely new branch of pharmaceutics in which small, orally available molecules are developed to increase circulating levels of one or more of the 28 known endogenous angiogenesis inhibitors in the body [26,27]. In this paper, I will outline this new concept and the evidence for it. Endostatin is a centerpiece of this argument because more research has been published on this protein than on any other endogenous angiogenesis inhibitor.

Discovery of endostatin

Endostatin [28,29] is a 20 kDa internal fragment of the carboxyterminus of collagen XVIII [30,31]. It was discovered by Michael O'Reilly in the Folkman laboratory based on Folkman's hypothesis of a mechanism to explain the phenomenon that surgical removal of certain tumors leads to rapid growth of remote metastases. This hypothesis also initiated the prior discovery of angiostatin [32] in the same laboratory. In its simplest terms, this hypothesis said that "if tumors produce both stimulators and inhibitors of angiogenesis, the stimulators (i.e., VEGF, bFGF) could accumulate in excess of inhibitors *within* an angiogenic tumor. In the *circulation* however, the ratio would be reversed. Angiogenesis inhibitors would increase relative to stimulators, because of rapid clearance of stimulators from the blood." (VEGF has a half-life of ~3.5 min in the circulation.) Folkman formulated this hypothesis after reading Noel Bouck's first report in 1989 that the emergence of tumor angiogenesis was the result of a shift in balance between positive and negative regulators of angiogenesis in a tumor [33]. Endostatin is the first endogenous inhibitor of angiogenesis to be identified in a matrix protein.

Early problems

(a) Difficulties with production

Endostatin protein was initially purified from the urine of tumor-bearing mice, providing a few micrograms for analysis of amino acid sequence [28]. Recombinant mouse endostatin was then produced in *E. coli*. Endotoxin was removed by polymyxin chromatography. However, resolubilization methods at the time gave very low yields of active protein (~1–2%) that were insufficient for testing antiangiogenic and antitumor activity in vivo, but insoluble purified endostatin had the consistency of toothpaste. To overcome this impasse, insoluble endostatin was injected subcutaneously in mice. A white deposit formed (of approximately 2–3 mm diameter) that slowly disappeared over 2–3 days. The antitumor activity was dramatic. Murine tumors could be completely regressed as long as the mice received a daily injection of the insoluble endostatin [29]. Furthermore, discontinuation of endostatin therapy was followed by recurrence of tumor growth, but tumors could be completely and repeatedly regressed by resumption of endostatin therapy. This result demonstrated absence of acquired drug resistance and absence of toxicity, even when therapy was continued for more than 100 days. A surprising result in three out of three different tumor types was that, after prolonged endostatin therapy, tumors did not recur but remained dormant at a microscopic size throughout the normal lifetime of the mice. The mechanism of this sustained dormancy is unclear. There was general criticism of the insoluble endostatin experiments by reviewers and skeptical colleagues, who felt that insoluble endostatin protein was denatured. They ascribed the antitumor activity to contamination with bacterial endotoxin, despite the fact that the preparations were endotoxin-free.

(b) Difficulties in reproduction

When other laboratories tried to make their own recombinant endostatin from *E. coli*, there were incon-

sistencies of endostatin activity [34]. Furthermore, when the Folkman laboratory mailed active endostatin, *E.-coli*-derived preparations to colleagues, the insoluble *E. coli* preparations were not always active. Recently, Kashi Javaherian and Robert Tjin in the Folkman laboratory reported that "the entire antitumor, antiendothelial migration, and antipermeability activities of endostatin are mimicked by a 27-amino-acid peptide corresponding to the NH_2-terminal domain of endostatin" [35]. It is acid-resistant. Others have previously reported endostatin peptides [36–40].

"Aggregation of endostatin in *E. coli* preparations is caused by random intermolecular disulfides after PBS dialysis." While endostatin reveals "a single protein molecule under reducing conditions, most of the protein in an identical sample does not enter the polyacrylamide gel under nonreducing conditions. It is probably the degree of nonspecific aggregation that is responsible for the lack of activity in some of the earlier *E. coli* preparations" [35]. In animals, endostatin is most likely released in a sustained manner from the subcutaneously injected aggregate, resulting in "a denatured protein or partial fragments," which have antitumor activity due to their NH_2-terminal peptide. Some of the early *E. coli* preparations yielded larger aggregates which were inefficiently released or were inactive. This problem was solved by production of soluble endostatin (human and murine) in yeast (*Pichia pastoris*). Currently, virtually all laboratories around the world now produce their own recombinant endostatin from yeast or have used Entremed's soluble human recombinant endostatin from yeast.

However, Gorelik [41] obtained the *E. coli* plasmid from the Folkman laboratory, generated soluble endostatin at a yield of 150 mg/l and 99% purity and solubilized it by refolding the protein. He treated Lewis lung carcinomas with 20 mg/kg/day and obtained >99% tumor inhibition and also complete regression, thus reproducing O'Reilly and Folkman's original study with *E. coli* endostatin [28,29]. Furthermore, Perletti et al. in Milan purified rat endostatin from *E. coli* and treated spontaneous rat mammary carcinoma induced by a carcinogen [42]. Tumor regression was complete and yielded the same residual microscopic dormant nodules observed by O'Reilly and Folkman [28,29]. Furthermore, human recombinant endostatin recently approved in China [3] for the treatment of lung cancer is made from *E. coli* and is refolded to achieve a soluble product.

Soluble recombinant endostatin from yeast showed significant antiangiogenic and antitumor activity in mice but did not induce the complete tumor regressions previously observed with insoluble *E. coli* preparations, unless soluble endostatin was administered *continuously* by an implanted micro-osmotic pump [44,45] (Fig. 1). These results emphasized the importance of continuously elevated circulating levels of endostatin to achieve *optimum inhibition and regression of tumors*.

(c) The zinc controversy

Another problem was whether zinc binding by endostatin is necessary for its antiangiogenic and antitumor activity. A year after the first report of endostatin, Thomas Boehm in the Folkman laboratory showed that replacement of histidines 1 and 3 by alanines blocked the antitumor activity of endostatin [46]. This finding was challenged by two later reports [47,48]. In one report [47], a mutant endostatin was prepared by deleting five amino acids in the COOH and NH_2-termini. This construct appeared to have the same antitumor activity as full-length endostatin. However, in the renal cell carcinoma model employed, endostatin was administered only at the periphery of the tumor, and the injection dosage was only 10 micrograms/kg/day for 4 days. Endostatin was administered when the tumor size was 300 mm^3 and lasted for only 4 days when the tumor size reached 500 mm^3. In contrast, in our experiments, endostatin was administered systemically and was not injected into the periphery of the tumor. We initiated treatment of Lewis lung carcinoma at 100 mm^3 and continued until the untreated controls were ~6000 to 7000 mm^3. In another report [48], removal of 4 amino acids HSHR, from the NH_2-terminus, did not affect its antitumor activity. Measurements of Zn binding revealed that this mutant bound 2 atoms of Zn per molecule of

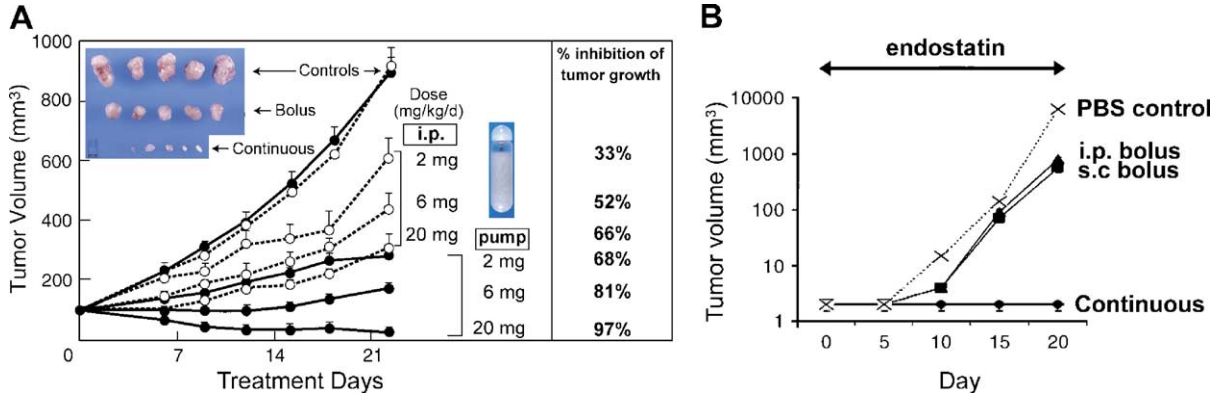

Fig. 1. ~~Continuously elevated blood levels of an angiogenesis inhibitor provide more effective antitumor activity than peaks and valleys [43,45].~~

endostatin, whereas the wild-type bound 10 atoms of Zn per endostatin molecule. This finding is problematic because, in our crystal structure analysis of endostatin, the molecule contains one atom of zinc/endostatin molecule, and the removal of the four amino acids HSHR from the NH_2-terminus results in loss of zinc binding [49]. Other reports of peptides of endostatin that do not bind zinc, but were not tested against tumors, or were not compared at equimolar concentrations are discussed in [35], but not here to save space. The definitive experiments by Tjin and Javaherian [35] show that the 27-amino-acid peptide corresponding to the NH_2-terminal domain of endostatin contains three histidines that are responsible for zinc binding. Mutations of the zinc-binding histidines abolished its antitumor and antiendothelial migration activities, but not its antipermeability activity.

Because endostatin is generated by proteolytic cleavage of collagen XVIII [50,51], the first amino acid at the NH_2-terminus of endostatin is a histidine. Because the presence of histidine confers zinc binding to endostatin, we conclude that the processing of endostatin may be highly regulated.

Table 2A
Tumors significantly inhibited by recombinant endostatin protein

Tumor	Reference
Murine tumors	
Ovarian	[52]
Acute myelogenous leukemia (chloroma)	[53]
Colorectal carcinoma	[54]
Spontaneous mammary carcinoma	[56]
B-16 melanoma	[60]
Transplantable mammary carcinoma	[62]
Hepatoma	[101]
Lung adenocarcinoma	[70]
Lewis lung carcinoma	[41]
Rat glioma in brain (continuous endostatin + PKC)	[102]
Murine colorectal liver metastases	[103]
B-16 melanoma + endostatin fusion with angiostatin	[104]
Human myeloid leukemia in SCID rats	[105]
Lewis lung carcinoma	[106]
Rat carcinogen induced mammary cancer	[42]
Pancreatic insulinoma	[107]
Human tumors in mice	
Laryngeal squamous cell carcinoma	[55]
Glioblastoma (U87)	[56]
Prostate carcinoma (PC3)	[59]
Neuroblastoma	[61]
Testicular carcinoma	[64]
Breast carcinoma	[65]
Head and neck squamous cell carcinoma	[66]
Kaposi's sarcoma	[67]
Pancreatic carcinoma	[68]
Human non-small-cell lung cancer	[108]
Human pancreatic carcinoma	[45]
Brain tumors (U87)	[109]
Non-Hodgkin lymphoma (high grade)	[110]
Renal cell cancer	[111]
Bladder cancer	[112]
Murine metastatic tumors	
Lung adenocarcinoma (completely inhibited)	

A few are discussed in detail in text.

Antitumor activity by endostatin protein

Many animal and human tumors in mice have been inhibited by administration of endostatin protein in studies from different investigators (summarized in Table 2A). A few reports selected from more than 100 which show significant antitumor activity by endostatin are summarized below in order to illustrate certain principles of endostatin's therapeutic use. These examples also demonstrate some of the different types of murine and human tumors treated, as well as the different doses, schedules and routes of administration, employed by different investigators. All reports emphasize the lack of toxicity. Also endostatin does not interfere with wound healing. Tumor responses ranged from 47% to 91% inhibition by endostatin doses of 10 mg/kg to 100 mg/kg/day. Human ovarian cancer in nude athymic mice was inhibited by 73% by NGR-endostatin by day 41 compared to 58% inhibition of tumor growth with native endostatin administered at 20 mg/kg/day [52]. NGR-endostatin is a recombinant human endostatin (from yeast) genetically modified to contain an asparagine–glycine–arginine sequence (NGR) which is known to home to blood vessels because endothelial cells express high levels of aminopeptidase N. Murine ovarian cancer was also significantly inhibited.

A murine acute myelogenous leukemia (chloroma) in SCID mice was inhibited by 73% after 4 weeks by murine endostatin continuously released from microencapsulated cells transfected with murine recombinant endostatin (yeast) endostatin [53]. Survival was increased by 30% ($P \leq 0.003$), and microvessel density was significantly decreased. Endostatin in serum was 50 ng/ml in wild-type mice vs. 80 ng/ml in treated mice. Endostatin had no effect on the proliferation of tumor cells in vitro. In other mouse strains, serum endostatin levels have been reported as low as 10–15 ng/ml.

Liver metastases from murine colorectal cancer cells injected into the spleen were prevented by a 2 h pre-treatment in vivo with endostatin [54]. The dose of endostatin at 500 μg s.c. per day for 19 days is approximately equivalent to 25 mg/kg/day.

Human laryngeal squamous cell carcinoma in nude mice was inhibited by 45.9% ($P < 0.01$) at an endostatin dose of 20 mg/kg × 21 days. Intratumoral microvessel density was significantly decreased [55].

Spontaneous mammary carcinoma in transgenic mice was significantly inhibited by a mutated form of recombinant human endostatin [56].

Human brain tumors (U87) in a transparent chamber in the skulls of nude mice were inhibited by 74% (reduction in tumor volume) by direct microinfusion of endostatin for 3 weeks, at 2 mg/kg/day ($P = 0.05\%$). Microvessel density

was decreased by 33.5% ($P \leq 0.005$), and there was a 3-fold increase in tumor cell apoptosis ($P \leq 0.002$) [57]. Survival increased significantly ($P < 0.003$) and was dose-dependent.

In a model of human blood vascular cells which developed into hemangioma-like lesions in immunodeficient mice, endostatin inhibited these vascular lesions by 95% in 20 days [58]. Pericyte recruitment was inhibited by 35%.

Human prostate cancer (PC3), and human glioblastoma U87, in nude athymic mice at low dose (0.75 mg/kg/day) was administered q12 h for 14 days [59]. Tumor growth delay was significant for endostatin alone and for another angiogenesis inhibitor SU5416 and was increased 3- to 4-fold by both together ($P \leq 0.01$). This experiment illustrates the increased efficacy of broadening the spectrum of antiangiogenic activity. Functional vessel density was also decreased.

Murine melanoma (B16) in the foot pad was inhibited by 77% by human full-length endostatin, but only by 55% by a truncated human endostatin with deletion of N- and C-termini [60]. There was apparently no zinc binding in the truncated endostatin because of the absent histidine. However, lung metastases were inhibited more potently by the truncated endostatin than by full-length endostatin by i.p. injection of 0.3 mg/kg/day, after removal of a 1000 mm^3 tumor from the subcutaneous dorsum. These results suggest that zinc binding is not necessary for antitumor activity, but the peptide and the full-length endostatin were not tested at equimolar concentrations. Furthermore, it is not clear if the injection was intratumoral.

Human neuroblastoma was inhibited by 47% when human endostatin was administered subcutaneously at 10 mg/kg/day in nude mice for 10 days [61]. But, tumor inhibition was 61% when endostatin was administered continuously by an implanted pump at only 30% of the subcutaneous daily dose. These data further emphasize the improved efficacy from continuous delivery.

Murine mammary carcinoma implanted orthotopically was inhibited by endostatin administered at 50 mg/kg/day subcutaneously, and it synergized adriamycin without the increasing cardiotoxicity observed when other anti-cancer drugs are added to adriamycin [62].

Murine hepatoma was inhibited by subcutaneous administration of solubilized recombinant endostatin from *E. coli*. Microvessel density was significantly decreased, and tumor necrosis was increased [63].

Human testicular cancer was treated with endostatin administered at 10 mg/kg/day continuously by micro-osmotic pumps implanted subcutaneously.

Endostatin alone, carboplatin alone or thrombospondin-1 alone had *no* effect on the growth of the primary tumor or on metastases that occurred in all animals by 6 months [64]. However, a combination of endostatin plus thrombospondin-1, or a combination of endostatin plus carboplatin, *prevented all metastases*, significantly inhibited primary tumors, decreased vascularity, decreased tumor cell expression of VEGF-A and increased tumor cell apoptosis. These results emphasize another important principle: a tumor refractory to three drugs administered as single agents can become responsive to a combination of two angiogenesis inhibitors or to a combination of an angiogenesis inhibitor and a cytotoxic chemotherapeutic agent.

Human breast cancer in nude mice was inhibited by 80% when treated with a novel fusion protein of endostatin (at only 5 mg/kg/day) compared to 60% for endostatin alone [65]. This is another example that increasing the half-life of circulating endostatin increases its efficacy.

Human head and neck squamous cell carcinoma was significantly inhibited by endostatin therapy. However, endostatin also inhibited tumor cells directly by suppressing tumor cell migration and invasion, as well as by down-regulating gene expression of several pro-migratory molecules and upregulating AP-1 in the tumor cells. This is a first demonstration that, for some tumors, endostatin's clinical efficacy may extend beyond its antiangiogenic activity and include antitumorigenic activity as well, yet without toxicity to other tissues [66].

AIDS-related Kaposi's sarcoma is another example in which endostatin has antitumor and antiangiogenic activities. The tumor cells internalize endostatin which co-localizes to tropomysin microfilaments and inhibits cytokine-mediated migration and invasion of tumor cells [67].

Human pancreatic cancer in SCID mice was treated for 21 days by human recombinant endostatin administered subcutaneously at a relatively high dose (100 mg/kg/day) compared to other reports in the literature. A slowly growing pancreatic cancer (BXPC3) was inhibited by 91%. In contrast, the same dose inhibited the rapidly growing variant of this tumor (ASPC-1) by only 69% [68]. Endostatin significantly suppressed microvessel density by 66% ($P < 0.001$ for both tumors). This pair of tumors illustrates a general rule that slowly growing tumors are easier to treat with antiangiogenic therapy than are rapidly growing tumors, i.e., just the opposite of cytotoxic chemotherapy. This has also been reported for a pair of human bladder cancers one of which grows 10 times faster than the other [69]. For more rapidly growing tumors, higher doses of antiangiogenic therapy are generally required.

A murine lung adenocarcinoma (LA795) growing subcutaneously in mice was treated with endostatin at 20 mg/kg/day. The primary tumors were inhibited, and lung metastases were completely inhibited (100%), in contrast to control mice (PBS treated) with widespread metastases. Microvessel density was decreased ($P \leq 0.01$) [70].

There are three reports of lack of antitumor activity by endostatin protein. For example, a Morris hepatoma in rat liver did not respond to endostatin infusion into the hepatic artery, together with mitomycin C, plus lipiodol plus immunotherapy. The experiment was short-term and limited by the catheter life.

Antitumor therapy by endostatin gene therapy

Endostatin appears to be an ideal candidate for gene therapy. It is a highly conserved protein and in evolution is found as early as *C. elegans*. It has shown virtually no toxicity in animals or in patients, including four patients who have received endostatin daily for >3.5 years. In more than 60 reports since 1997, endostatin gene therapy of the full-length protein has significantly inhibited growth of primary tumors and their metastases. In animal studies, inhibition was up to 86% reduction in tumor volume and/or complete prevention of pulmonary metastases. Lowest inhibition of tumor growth was ~40–45% (Table 2B). In transgenic mice overexpressing endostatin, a small increase in circulating endostatin of approximately 1.6-fold is sufficient to confer dramatic protection against tumor growth [71]. In individuals with Down syndrome, a similar small increase of circulating endostatin is associated in part with broad protection against the majority of human tumors [72]. The recent report that all of the antitumor activity of endostatin is mediated by a 27-amino-acid N-terminal fragment of endostatin provides an expanded opportunity for future gene therapy with endostatin [35]. Below are representative examples of certain principles of endostatin gene therapy and of certain advantages over endostatin protein therapy (see also Table 2B).

When mouse endostatin was transfected into mouse renal cell carcinoma or human colon cancer cells so that endostatin was constitutively secreted, flank tumors were inhibited by 73–91% and liver and lung metastases were prevented or significantly inhibited [73]. Inoculation of a cell mixture containing only 25% endostatin-transfected tumor cells with 75% control tumor cells inhibited growth of flank tumors as effectively as 100% of endostatin-transfected cells [73]. This result suggests that "gene delivery of endostatin into even a minority of tumor cells may be an effective strategy to prevent progression of micrometastases to macroscopic disease."

Mouse brain tumor cells (C6 glioma) transfected with endostatin resulted in 71% inhibition of growth of orthotopic brain tumors implanted into brains of nude immunodeficient mice or in rats accompanied by a 50% decrease in microvessel density [74]. Complete tumor inhibition or dormancy was not observed in these mice. These results suggest that "endostatin could be developed as an adjuvant gene therapy for the treatment of brain tumors."

However, systemically administered antiangiogenic therapy may need to accompany endostatin gene therapy (for example, Caplostatin [75]).

In contrast to endostatin gene therapy alone, when endostatin gene therapy was combined with intratumoral adenovirus-mediated herpes simplex virus thymidine kinase, a cytotoxic virus, orthotopic renal cell cancer remained permanently dormant and was eradicated in 57% of treated mice [76]. This result indicates that cytotoxic gene therapy may be synergized by endostatin gene therapy.

When mouse breast cancer was implanted orthotopically into the mammary fat pad, it metastasized to brain. Intramuscular endostatin gene therapy increased the circulating levels of endostatin from the normal of 5 ng/ml (4.5–6.4 ng/ml) to a peak of 17.8 ng/ml (14.5–20.7 ng/ml) and inhibited the brain metastases by ~60%, but it had no effect on growth of the primary breast cancer [77]. This study shows that a rise in circulating endostatin level of only 3.5-fold is sufficient to inhibit growth of brain metastasis, but not the primary tumor. This relatively low level of increased endostatin reveals a differential effect on the primary vs. its metastasis that may present when endostatin therapeutic levels are borderline.

In a remarkable study of gene therapy of human glioblastoma xenografts in nude mice, a combination of 3 angiogenesis inhibitors was administered by intratumoral injection of plasmids containing two constructs, an angiostatin-endostatin fusion gene (statin-AE) and a soluble vascular endothelial growth factor receptor (sFlt-1) [78]. There was significant reduction in tumor microvessel density. Tumors were eliminated in up to 50% of mice ($P = 0.003$). Survival was prolonged by up to 4-fold ($P = 0.008$). Fifty percent of mice were still living at the end of the experiment (200 days). Intracranial and subcutaneous tumors were both successfully treated. These results show the advantage of combinatorial antiangiogenic gene therapy, especially for brain tumors. They also illustrate the advantage of intratumoral antiangiogenic gene therapy over systemic administration, at least for localized brain tumors, because gene transfer can facilitate sustained levels of inhibitor at the tumor site. Intratumoral antiangiogenic therapy of brain tumors may also produce a reverse diffusion of inhibitor toward neighboring capillaries.

Endostatin gene therapy enhanced the effect of ionizing radiation in Lewis lung carcinomas [79]. Tumor volumes were up to 50% smaller with the combination therapy. These results point to a possible future role for antiangiogenic gene therapy as a potentiator of ionizing radiation.

Endostatin gene therapy also enhanced the antitumor effect of gemcitabine and produced a significant decrease of tumor volume and of vascularization without added toxicity in a human lung cancer model in mice [80].

Several novel approaches for administering endostatin gene therapy have been reported. Intra-arterial delivery of endostatin gene therapy to rat brain tumors resulted in an 80% reduction in tumor volume, an enhanced survival time up to 47%, and a 40% decrease in number of tumor vessels [81]. Oral delivery of endostatin gene therapy by a unique bacterial carrier inhibited liver tumors in mice [82]. In certain colon cancers (murine C51, human HT29), endostatin directly inhibited the tumor cells in addition to its antiangiogenic activity.

Table 2B
Tumors significantly inhibited by endostatin gene therapy

Tumor	Reference
Murine primary tumors	
Renal cell carcinoma	[73]
Renal cell carcinoma	[113]
Brain tumors	[74]
Renal cell carcinoma	[114]
Renal cell carcinoma	[76]
Breast cancer and brain tumor (FM3A P-15) metastasis	[77]
Breast cancer (mid-T2-1)	[115]
Breast cancer (spontaneous)	[116]
Lewis lung carcinoma	[117]
~~Lewis lung carcinoma~~	~~[118]~~
Lewis lung carcinoma	[119]
Lewis lung carcinoma	[120]
Lewis lung carcinoma	[114]
Leukemia (L1210)	[113]
Myeloproliferative disease (resembling human chronic myelogenous leukemia)	[121]
Melanoma (K1735)	[122]
Melanoma (B16F10)	[123]
Bladder MBT-2	[123]
Colon cancer (colon 26)	[124]
Colon adenocarcinoma MC38	[125]
Hepatocarcinoma (H22)	[126]
Hepatoma (Hepa1c1c7)	[127]
Hepatocarcinoma	[101]
Melanoma (B16F10) (and metastases)	[128]
Spontaneous tongue carcinoma	[129]
Spontaneous breast cancer in C3(1)/T mice	[56]
Mammary carcinoma MCa-4	[130]
Brain tumor	[131]
Murine mammary ascites (TA3)	[132]
Neuroblastoma NXS2	[133]
Murine pulmonary metastases	
Fibrosarcoma	[134]
Fibrosarcoma (NFsa Y83)	[135]
Melanoma (B16F10)	[136]
Rat tumors	
Morris hepatoma	[137]
Hepatoma (orthotopic)	[138]
Gliosarcoma (9L)	[81]
Osteosarcoma	[139]
Hamster	
Pancreatic cancer (orthotopic) and liver metastases	[126]
Human tumors in mice	
Colon cancer (SW620)	[73]
Colorectal cancer (HT29)	[140]
Colorectal cancer (HT29)	[141]
Colorectal	[142]
Colorectal advanced stage IV (T3N1M1)	[143]
Colorectal cancer (LoVo)	[144]
Glioblastoma	[145]
Lung cancer	[80]
Non-small-cell lung cancer (KNS 62) (and metastases)	[146]
Hepatocellular carcinoma	[80]
Hepatocellular carcinoma (BEL-7402)	[147]
Hepatocellular carcinoma	[148]
Hepatocellular carcinoma Hep3B	[149]
~~Hepatocellular carcinoma HepG2~~	~~[82]~~
Hepatocellular carcinoma HepGH	[43]
Hepatocellular carcinoma HepG2	[82]
Hepatocellular carcinoma HepG2	[150]
Hepatocellular carcinoma	[151]
Hepatocellular carcinoma (SMMC7721)	[152]
Ovarian cancer	[153]
Ovarian carcinoma	[154]
Ovarian carcinoma (SKOV3)	[155]
Ovarian carcinoma (SkOV3)	[156]
Tongue squamous cell carcinoma	[157]
Bladder carcinoma (KU-7) orthotopic	[158]
Lack of inhibition of angiogenesis, tumor growth and/or metastases	
Murine primary tumors	
Fibrosarcoma T241	[84]
Murine lung cancer	[89]
Lewis lung carcinoma (weak antitumor activity)	[83]
Human tumors	
Acute lymphocytic leukemia	[86]
Breast cancer (MDA-MB-231) (minimal effect)	[115]
Neuroblastoma (SKNAS)	[87]

A few are discussed in detail in text.

In 6 reports, endostatin gene therapy failed to inhibit tumor growth (Table 2B). In Kuo et al. [83] (from the Folkman lab) and in Pawliuk et al. [84], one explanation is that the circulating endostatin levels were too high. Since these papers were published, it has been found that endostatin antiangiogenic and antitumor efficacy is biphasic and operates over a U-shaped curve [85] (Fig. 2). Circulating levels of endostatin that are too high or too low are inactive. The normal range of endostatin in mouse blood among a wide variety of reports is ~5–15 ng/ml. Effective therapeutic levels are up to ~80–450 ng/ml. Higher levels may be less effective. In the papers by ~~Eisterer et al. [86] and by~~ Jouanneau et al. [87], a possible explanation for endostatin's failure is variable aggregation of recombinant endostatin from *E. coli*, as discussed by Tjin et al. [35]. Other explanations are suggested by Steele [88]. Cui et al. [89] reported the very unusual upregulation of VEGF secretion from tumor cells by endostatin, and this may have overcome the antiangiogenic activity of endostatin. Tumors from these cells became hypervascularized and grew more rapidly instead of regressing as Lewis lung carcinoma did in all previous reports. The mechanism is unknown, although incubation of the tumor cells with endostatin did not elicit VEGF secretion and incubation of the gene transfected tumor cells with antibody to endostatin failed to stop VEGF secretion (Fig. 2).

Indraccolo has written a very thoughtful review of antiangiogenic gene therapy [90].

Mechanisms of the antiangiogenic activity of endostatin

Endostatin was discovered by employing the same strategy that led to the earlier discovery of angiostatin

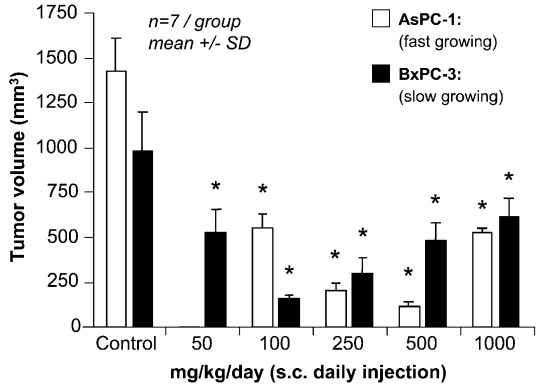

Fig. 2. Biphasic, U-shaped, dose response curve of endostatin. The dose and efficacy of certain endogenous (interferon alpha) and synthetic (rosiglitazone) angiogenesis inhibitors follow a biphasic, U-shaped relationship.

[91], i.e., isolation of an antiangiogenic protein from urine of tumor-bearing mice in which metastases are suppressed until the primary tumor is removed surgically. One of the major mechanisms of action of angiostatin, which explains most of its antiangiogenic functions, is based on its interaction with ATPase on the endothelial surface [92]. A second mechanism is angiomotin [93]. For endostatin, unlike angiostatin, a mechanism of action has not been distilled to a few signaling pathways. ~~The mechanism of action of endostatin has been researched intensely since the report of its discovery in 1997 [28].~~ The clear picture to emerge is the pervasiveness of its influence—one that may be attributable in part to a long presence of the gene over the evolutionary course of the human genome. Several unique activities have been reported for endostatin since 1997. Endostatin binds $alpha_5beta_1$ integrin on endothelium [94,95]. For antiangiogenic activity, endostatin appears to be dependent on binding to E-selectin [96]. Also, endostatin blocks activity of metalloproteinases 2, 9, and 13 [97].

Shichiri and Hirata [98] showed that endostatin-initiated intracellular signaling in endothelial cells caused downregulation of a set of growth-associated genes in a wide range of endothelial lineage cells. Abdollahi et al. [25,18] shed light on why the influence of endostatin is so extensive, and what this says about mechanism. Using custom microarrays covering over 90% of the human genome, they reported that ~12% of all genes are significantly regulated in human microvascular endothelial cells exposed to endostatin. They noted that the upregulated genes as a group include the known angiogenesis inhibitors, while the downregulated genes include the known stimulators. Revealed is a networked action of endostatin that cannot be reduced to single gene responses. To fully answer the question of mechanism, then it is necessary to think beyond individual molecular regulations and consider common physiological responses. On hundreds of genetic pathway fronts, endostatin is shown to suppress one physiological process — angiogenesis. From this perspective, it is not so surprising that the mechanism of endostatin has not been distilled to a few signaling cascades. More surprising is that there are so many genetic routes to angiogenesis suppression and that a single molecule can regulate these routes en masse to control, e.g., the angiogenic switch in tumors. In fact, a review of all published papers to date shows that endostatin suppresses mainly pathological angiogenesis and appears to have little or no activity against wound healing or reproduction. This phenomenon is not yet explained except for the possibility that certain pathological forms of angiogenesis are associated with upregulation of integrins (i.e., $alpha_5beta_1$) [71,94,95] or E-selectin [96] (Table 3).

Table 3
Endogenous angiogenesis inhibitors (Adapted from Nyberg et ~~al. [97]~~).

Percent Down		Fold Up	
67%	VEGF-A	1.6	Maspin
72%	Neuropilin-1	1.7	DSCR5
66%	bFGF	6.0	IFN-g R1
44%	FGF-R1	2.7	Cdk4 inhib p18
50%	FGF-R2	2.2	Thrombspodin-1
55%	HGF	6.0	Sphingomyelinase
55%	EGFR	1.5	AT-III
74%	HIF-1 alpha	2.0	Kininogen
60%	HIF1 Resp	2.8	STAT inh. (PIAS)
86%	Fibronectin	15	Notch 2
72%	Id1	4.0	Ephrin B3
60%	Id4	3.9	Ephrin A3
58%	c-myc	7.2	Collagen XVI a1
79%	c-fos	3.4	Adenomatosis Polyposis Coli
76%	Ephrin-A1	2.45	HIF-1α inhibitor (HIF1AN)
41%	NFKB p65	2.1	PDCD5 (Program cell death)
23%	bcl-2	2.7	SPOCK (sparc/osteonection)
41%	TNF R-1		
81%	JUNB		
41%	cox-2		
63%	P-selectin		

Endostatin

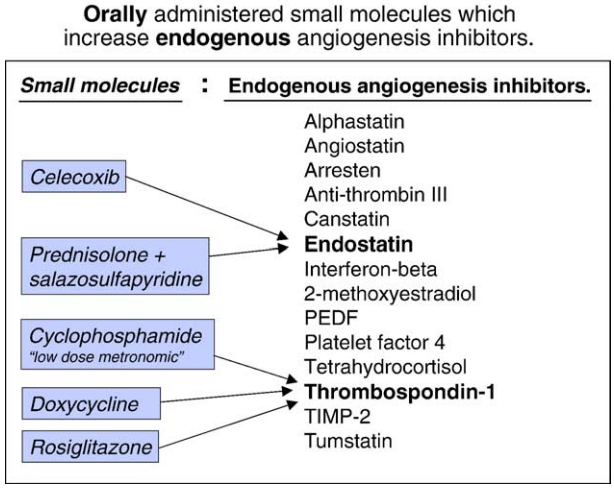

Fig. 3. Certain small molecules can induce elevations of endogenous angiogenesis inhibitors. These results suggest the possibility of a new pharmaceutical field.

Summary and future directions

At this writing, at least 28 endogenous angiogenesis inhibitors have been discovered in plasma and/or in extracellular matrix [26,97]. Endostatin is the first endogenous angiogenesis inhibitor to be discovered as a fragment of the extracellular matrix. It is the most studied of the endogenous angiogenesis inhibitors. Kalluri showed that elevating the circulating level of endostatin (by genetic overexpression in endothelium) by less than 2-fold can suppress tumor growth by 2- to 3-fold [71]. It is possible that elevation of two or more endogenous inhibitors could possibly suppress tumor growth even more effectively or prevent it completely.

Several reports suggest that certain small molecules that can be taken orally will raise the endogenous expression of specific angiogenesis inhibitors or raise their plasma or serum level perhaps by alternative means, such as mobilization from matrix or platelets. For example, celecoxcib can increase serum endostatin [99]. Prednisolone and salazosulfapyridine can increase the endostatin level in joint fluid [100]. Doxycycline [5] and rosiglitazone can increase expression of thrombospondin-1. These are illustrated in Fig. 3. A possible new pharmaceutical field could be developed around the future discovery of low molecular weight, orally available drugs that could increase endogenous angiogenesis inhibitors to protect against cancer as well as other angiogenesis-dependent diseases. This would help to broaden antiangiogenic therapy of cancer. Endostatin is a paradigm of a broad spectrum endogenous antiangiogenic molecule.

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Characterization of blood derived endothelial progenitor cells for tissue engineering applications

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ABSTRACT

Endothelial progenitor cells (EPC) in peripheral blood represent a promising cell source to non-invasively obtain a robust endothelial population for cardiovascular tissue-engineering applications. However, due to the inherent heterogeneity of blood preparations, both phenotypical and functional characterization, as well as the evaluation of cell expansion capability, must be performed. Towards this end, EPC were isolated from the mononuclear cell fraction of human umbilical cord blood by positive selection of CD31+ cells. The endothelial phenotype was confirmed by FACS, indirect immunofluorescence and RT-PCR. EPC were uniformly positive for CD31, VE-cadherin, vWF, CD105, CD146, KDR and eNOS, while negative for the haematopoietic and monocytic markers CD45 and CD14. The potential of these EPCs for vasculogenic or angiogenic function was demonstrated the superior proliferative and migratory responses to VEGF and bFGF when compared to human dermal microvascular endothelial cells (HDMEC). EPC were routinely expanded for more than 65 population doublings such that 10^{11} endothelial cells were obtained from 25 mL of cord blood within 30 days of culture, an amount more than sufficient for any autologous tissue engineering application. As the EPC were expanded in culture, the morphology, growth kinetics and proliferative responses toward angiogenic growth factors progressively resembled those of mature human microvascular endothelial cells, indicating a process of in vitro cell maturation over time. We hypothesize that EPCs at different stages of ex vivo expansion will be required for specific tissue-engineering applications depending on the functional and phenotypic properties needed to achieve the appropriate degree of vascular development.